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Abstract

Tyrosine phenol-lyase (TPL), which catalyzes the beta-elimination reaction of L-tyrosine, and aspartate aminotransferase (AspAT), which catalyzes the reversible transfer of an amino group from dicarboxylic amino acids to oxo acids, both belong to the alpha-family of vitamin B6-dependent enzymes. To switch the substrate specificity of TPL from L-tyrosine to dicarboxylic amino acids, two amino acid residues of AspAT, thought to be important for the recognition of dicarboxylic substrates, were grafted into the active site of TPL. Homology modeling and molecular dynamics identified Val-283 in TPL to match Arg-292 in AspAT, which binds the distal carboxylate group of substrates and is conserved among all known AspATs. Arg-100 in TPL was found to correspond to Thr-109 in AspAT, which interacts with the phosphate group of the coenzyme. The double mutation R100T/V283R of TPL increased the beta-elimination activity toward dicarboxylic amino acids at least 10(4)-fold. Dicarboxylic amino acids (L-aspartate, L-glutamate, and L-2-aminoadipate) were degraded to pyruvate, ammonia, and the respective monocarboxylic acids, e.g. formate in the case of L-aspartate. The activity toward L-aspartate (kcat = 0.21 s⁻¹) was two times higher than that toward L-tyrosine. beta-Elimination and transamination as a minor side reaction (kcat = 0.001 s⁻¹) were the only reactions observed. Thus, TPL R100T/V283R accepts dicarboxylic amino acids as substrates without significant change in its reaction specificity. Dicarboxylic amino acid beta-lyase is an enzyme not found in nature.
Conversion of Tyrosine Phenol-lyase to Dicarboxylic Amino Acid β-Lyase, an Enzyme Not Found in Nature

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Tyrosine phenol-lyase (TPL), which catalyzes the β-elimination reaction of L-tyrosine, and aspartate aminotransferase (AspAT), which catalyzes the reversible transfer of an amino group from dicarboxylic amino acids to oxo acids, both belong to the α-family of vitamin B₆-dependent enzymes. To switch the substrate specificity of TPL from L-tyrosine to dicarboxylic amino acids, two amino acid residues of AspAT, thought to be important for the recognition of dicarboxylic substrates, were grafted into the active site of TPL. Homology modeling and molecular dynamics identified Val-283 in TPL to match Arg-292 in AspAT, which binds the distal carboxylate group of substrates and is conserved among all known AspATs. Arg-100 in TPL was found to correspond to Thr-109 in AspAT, which interacts with the phosphate group of the coenzyme. The double mutation R100T/V283R of TPL increased the β-elimination activity toward dicarboxylic amino acids at least 10⁴-fold. Dicarboxylic amino acids (L-aspartate, L-glutamate, and L-2-oxo acids) were degraded to pyruvate, ammonia, and the respective monocarboxylic acids, e.g. fromate in the case of L-aspartate. The activity toward L-aspartate (k₄₅ = 0.21 s⁻¹) was two times higher than that toward L-tyrosine. β-Elimination and transamination as a minor side reaction (k₉₄ = 0.001 s⁻¹) were the only reactions observed. Thus, TPL R100T/V283R accepts dicarboxylic amino acids as substrates without significant change in its reaction specificity. Dicarboxylic amino acid β-lyase is an enzyme not found in nature.

The pyridoxal 5’-phosphate-dependent enzymes (B₆ enzymes) catalyze a wide variety of reactions in the metabolism of amino acids (1). A comparison of amino acid sequences has shown that the majority of B₆ enzymes belong to the large α/γ-superfamily of homologous B₆ enzymes (2, 3). Tyrosine phenol-lyase (TPL) of Citrobacter freundii is a member of the α-family. It catalyzes the β-elimination of L-tyrosine to produce phenol, pyruvate, and ammonium (Equation 1).

\[
L\text{-Tyrosine} + H_2O \rightleftharpoons \text{phenol} + \text{pyruvate} + NH_3^+ \quad (\text{Eq. 1})
\]

A number of amino acids with suitable leaving groups on Cβ, such as L-serine and O-acetyl-L-serines (4), L-cysteine, 3-alkyl-L-cysteines (4, 5), and 3-chloro-L-alanine, are also substrates for β-elimination. Moreover, TPL has been found to catalyze markedly slower side reactions, i.e. β-replacement reactions (6, 7), racemization of alanine (8, 9), as well as transamination reactions of its substrates L-tyrosine, L-serine, and of the competitive inhibitors L-alanine, L-phenylalanine, and L-m-tyrosine (10).

X-ray crystallographic structure analysis has shown the folding pattern of the polypeptide chain of tetrameric TPL from C. freundii to be similar to that of dimeric aspartate aminotransferase (AspAT) (11), which, like TPL, is a member of the α-family of pyridoxal 5’-phosphate (PLP)-dependent enzymes. Despite their similarity in secondary and tertiary structure, the two enzymes show only low sequence identity, e.g. 28% between TPL of C. freundii and AspAT of Escherichia coli. AspAT catalyzes the reversible transamination reaction of the dicarboxylic L-amino acids aspartate and glutamate with the cognate 2-oxo acids 2-oxoglutarate and oxalacetate.

The structures of the active sites of TPL and AspAT are similar; most of the residues that participate in the binding of the coenzyme and the α-carboxylate group of the substrate in AspAT (12) are conserved in the structure of TPL (13). Obviously, these two homologous enzymes use the same protein scaffold to catalyze different reactions with different substrates. Thus, alteration of the specificity of a given enzyme by substitution of a limited number of critical amino acid residues seems feasible. Alignments of amino acid sequences of homologous enzymes may be used to identify the residues underlying the differences in their reaction and substrate specificity. Substitution of the residues to which the substrate binds has proven successful in changing the substrate specificity of several enzymes without destroying their catalytic apparatus (14–21).

This paper reports a homology modeling approach that, together with information obtained from structural and mechanistic studies of AspAT, was used to redesign the substrate specificity of TPL in favor of dicarboxylic amino acids.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis—**Plasmid pTZTPL (22) containing the entire coding sequence of C. freundii tyrosine phenol-lyase was used as template for in vitro mutagenesis. The tpl gene was amplified by the polymerase chain reaction using the following two synthetic oligonucleotides as primers: 5’-CGGCGTCTGACATAATTAT-TATTACATAGTATAGTAGAATATTATG-3’ and 5’-CCGAGATCTAATCCACTG-3’. The first oligonucleotide hybridizes to the 5’ part of the tpl gene and contains six histidine codons (italics), in frame, just before the stop codon and a new SalI site (underlined). The second oligonucleotide hybridizes to the unique BglII site (underlined) in the tpl gene upstream to the transcriptional start point. The resulting 1.9-kilobase pair polymerase chain reaction product was cut with BglII and SalI and subcloned into the BamHI-SalI sites of the expression vector pTZ18U (Bio-Rad) to generate pTZTPL-His.

The mutants were prepared by polymerase chain reaction from pTZTPL-His using the QuikChange™ Site-directed Mutagenesis Kit from STRATEGEN.
Stratagene and the following primer pairs: R100Ta, 5'-CCAATTGGCCGAGGCGACTCAGGCGGAGGCGCCGAGAAATCCCTG-3'; R100Tb, 5'-CTCGGGTCTCTGGCCTGGCTGGTATGAGG-3'; V283Ra, 5'-CTCGGTCTCCACAAGCGACTAAC-3'; and V283Rb, 5'-GTTACGTCCGTGTTATCAAGCAG-3'. The insertion of the histidine codons and the mutations was verified by cycle sequencing (Sequi Therm Long-Read Cycle Sequencing Kit-PC, Epicentre Technologies) with fluorescent primers using a DNA sequencer (LI-COR).

Expression and Purification—E. coli SVS270 cells were used as host for the pTZTPL-His and the mutant plasmids. The cells, grown as described previously (22), were thawed and suspended in 5 ml of Buffer A (50 mM sodium phosphate, 300 mM NaCl, 1 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 0.1 mM PLP, pH 8.0) per gram of wet weight. The cells were disrupted by three passages through a French press. Cell debris was removed by centrifugation at 25,000 × g at 4 °C for 30 min. The supernatant was passed through a 0.22-μm filter and directly applied onto a 13 × 1-cm column containing 2–3 ml of nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with Buffer A. The column was washed with Buffer A containing 20 mM imidazole until A280 of the flow-through solution was below 0.01. The TPL protein was then eluted with a 30-ml gradient from 20 to 250 mM imidazole in Buffer A. The pooled TPL fractions were dialyzed extensively against 0.1 M potassium phosphate, pH 7.0, containing 0.1 mM PLP, 1 mM EDTA, and 5 mM 2-mercaptoethanol. Purified wild-type and mutant TPLs were stable at least for 1 year when stored at −70 °C in the same buffer at a concentration of 2–5 mg/ml. All preparations were pure as indicated by SDS-polyacrylamide gel electrophoresis (10–15% PHAST-gel from Amersham Pharmacia Biotech).

Protein Determination—The concentration of purified TPLs was determined photometrically (ε280 = 8.37; Ref. 5) assuming a subunit molecular mass of 52.3 kDa (13) which takes into account the molecular mass of the His6 tag (0.84 kDa). The PLP content of the enzymes was determined from the absorption spectrum of the enzyme in 0.1 M NaOH, with a 8453 UV-visible diode-array spectrophotometer from Hewlett-Packard.

Measurement of β-Elimination Activity—The activity of the TPLs toward various amino acid substrates was measured using the coupled assay with lactate dehydrogenase and NADH previously described for tryptophan indole-lyase (24). The standard assay mixture contained 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 24 units of lactate dehydrogenase from bovine heart (Sigma), and varying concentrations of amino acid substrate in a final volume of 1 ml at 25 °C. The reaction was initiated by the addition of TPL and followed by the decrease in absorbance at 340 nm. Steady-state kinetic values of Kcat and kcat were obtained by fitting the data to the Michaelis-Menten equation using ORIGIN software (Microcal Software).
RESULTS

Design Strategy—In order to change the substrate specificity of TPL in favor of dicarboxylic amino acids, we compared TPL with AspAT using homology modeling and molecular dynamic simulations. The specificity of AspAT for dicarboxylic amino acids and oxo acids seems to be based primarily on the salt bridge-hydrogen bond interaction of the side chain of Arg-292 (of the adjacent subunit) with the distal carboxylate group of these substrates (12). In agreement with this notion, Arg-292 is conserved among all AspATs (29). Since the sequence identity between AspAT and TPL is too low (23%) to allow the use of standard alignment algorithms, comparison of their three-dimensional structures (13, 30) by superposition (Fig. 1) and with the program DALI (Ref. 31, 32; accessible through the World Wide Web at the following on-line address: http://www2.ebi.ac.uk/dali/) was used for analysis. The single mutant V283R enzyme, however, could not be expressed as soluble protein. The PLP content of the enzyme (Table I). The His-tagged TPL R100T/V283R enzyme and the single mutant TPL R100T were purified and expressed of the enzyme-substrate adduct did not appear to be optimum for a

The outer shell was then kept fixed, and another 2000 steps of minimization were applied. This was followed by a molecular dynamics simulation, which was initialized at 400 K for 1000 fs. After this initialization, the outer shell was again kept fixed. The simulation was continued for a total time of 20 ps. Every 100 fs the potential energy was analyzed. Within each picosecond, only the structure with the lowest potential energy was stored, resulting in a total of 20 low energy structures. All these 20 structures were then minimized for 2500 steps. The resulting minimized structures were found to be generally quite similar, and one of these corresponding to the average structure was chosen as starting point for all further simulations. The modeled structure of the wild-type enzyme with l-aspartate as substrate was obtained by replacement of l-tyrosine and applying the same minimization-dynamics procedure as for the wild-type structure with l-tyrosine as substrate. To model the double mutant enzyme, we replaced Arg-100 by a threonine and Val-283 by an arginine residue and applied again the minimization-dynamics procedure.

and Cγ of the amino acid substrate relative to the plane defined by the π system of the coenzyme-substrate adduct including Cβ (Scheme 1; Ref. 33). This notion agrees with previous studies by Faleev et al. (34) who have reported that aspartic and glutamic acid are not substrates but, in view of the low hydrophobicity of their side chains, anomalously strong inhibitors of TPL (Ki = 3.5 and 5.0 mM, respectively). We concluded that the introduction of an arginine residue into position 283 of TPL together with the substitution of Arg-100 with an uncharged residue, i.e. the double mutation R100T/V283R, might mimic the binding site for dicarboxylic substrates of AspAT and thus result in a corresponding alteration in the substrate specificity of TPL.

Expression of Wild-type TPL and Mutant TPLs—The C-terminal His6 tag did not interfere with the β-elimination activity of the enzyme (Table I). The His-tagged TPL R100T/V283R enzyme and the single mutant TPL R100T were purified and used for analysis. The single mutant V283R enzyme, however, could not be expressed as soluble protein. The PLP content of

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the mutant proteins was found to be 1 mol/mol of subunit, as has been shown previously for wild-type TPL (36). The UV-visible spectrum of the PLP form of the mutant enzymes is almost identical to that of the wild-type enzyme. Apparently, the topochemistry of the PLP-binding site is not significantly altered by the mutations.

Wild-type TPL in the presence of L-tyrosine exhibits a visible absorbance peak at about 500 nm attributable to the quinonoid intermediates formed by the elimination reaction with L-glutamate (Scheme 1; Ref. 8). Some other amino acids such as L- and D-alanine, L-phenylalanine, L-aspartic acid, L-methionine, and L-homoserine, which are not substrates for elimination, form stable quinonoid intermediates with wild-type TPL (34). TPL R100T also produced stable quinonoid intermediates upon addition of these amino acids. However, with TPL R100T/V283R no detectable quinonoid adduct was observed in the presence of any amino acid including L-tyrosine.

Changes in Substrate Specificity—TPL R100T/V283R and TPL R100T were tested for β-elimination activity toward L-tyrosine and dicarboxylic amino acids of various lengths (Table I). The k cat values of TPL R100T/V283R toward L-tyrosine was decreased 30-fold as compared with wild-type TPL without significant change in the K m value. When TPL R100T/V283R was tested for activity toward dicarboxylic amino acids using the coupled assay with lactate dehydrogenase and NADH, pyruvate was detected in the reaction mixtures. Thin layer chromatographic analyses confirmed the production of pyruvate. A yellow spot, the R f value of which was the same as that of authentic pyruvate, was detected on the plate as the unique product of the enzymic reactions with all dicarboxylic substrates. No 2-oxobutyric acid, which possibly might have been produced by a γ-elimination reaction of L-glutamate, was detected.

The expected products of the β-elimination reaction of the dicarboxylic substrates L-aspartate, L-glutamate, and L-2-aminoadipate are pyruvate, ammonia, and the monocarboxylic acid formate, acetate, and propionate, respectively. In the case of L-aspartate, formate was identified and determined using the coupled assay with formate dehydrogenase and NAD +. Equimolar amounts of pyruvate and formate were detected (Table II). Thus, TPL R100T/V283R catalyzes, in contrast to the wild-type enzyme, the β-elimination reaction of dicarboxylic substrates at least as efficiently as, or in the case of L-aspartate, even two times faster than that of L-tyrosine (k cat = 0.21 s −1; Table I). The K m value of TPL R100T/V283R for the β-elimination reaction with L-glutamate was significantly lower than the K m values with L-aspartate and L-2-aminoadipate. It seems that L-glutamate has the optimum size for binding among these dicarboxylic substrates. TPL R100T also catalyzed the β-elimination reaction of the dicarboxylic amino acids L-aspartate, L-glutamate, and L-2-aminoadipate; however, the reaction was up to six times slower than that with the double mutant enzyme. The K m values were also higher (up to 3 times, in the case of L-glutamate) as compared with the double mutant enzyme. Furthermore, the TPL R100T-catalyzed β-elimination reaction of L-tyrosine was five times faster than the TPL R100T/V283R-catalyzed reaction.

We also examined the reaction of TPL R100T/V283R and TPL R100T with 3-chloro-L-alanine, L-serine, S-methyl-L-cysteine, and S-(o-nitrophenyl)-L-cysteine which are good substrates for β-elimination by wild-type TPL (4, 5). The activity toward 3-chloro-L-alanine was only slightly lower than that of the wild-type enzyme (Table I). However, no measurable activity was observed with the other substrates. L-Cysteine sulfinate, which can be considered an analog of L-aspartate, was inert as substrate with both mutant TPL enzymes.

Changes in Reaction Specificity—TPL R100T/V283R was also tested for newly generated catalytic activities with a sen-
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Table II
Stoichiometry of the β-elimination reaction of tyrosine phenol-lyase
R100T/V283R with l-lysine

<table>
<thead>
<tr>
<th>Time of reaction (min)</th>
<th>Products</th>
<th>Formate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>44.1 μM</td>
<td>43.8 %</td>
</tr>
<tr>
<td>10</td>
<td>88.2 μM</td>
<td>87.7 %</td>
</tr>
</tbody>
</table>

*Pyruvate was measured using the coupled assay with lactate dehydrogenase and NADH.

Table III
Kinetic parameters for side reactions of TPL wild-type and TPL R100T/V283R

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activities</th>
<th>Wild-type</th>
<th>R100T/V283R</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Aspartate</td>
<td>Transamination</td>
<td>1.1 × 10⁻⁴</td>
<td>1.1 × 10⁻³</td>
</tr>
<tr>
<td>l-Glutamate</td>
<td>Transamination</td>
<td>1.3 × 10⁻⁴</td>
<td>1.5 × 10⁻³</td>
</tr>
<tr>
<td>l-Serine</td>
<td>Transamination</td>
<td>1.0 × 10⁻³</td>
<td>1.8 × 10⁻³</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>Transamination</td>
<td>1.5 × 10⁻³</td>
<td>2.1 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>Racemization</td>
<td>0.03</td>
<td>8 × 10⁻³</td>
</tr>
</tbody>
</table>

*Measured at a single substrate concentration of 250 μM for l-lysine, l-glutamate, l-serine, and of 100 μM for l-alanine.

Discussion

X-ray crystallographic analysis has identified Arg-292 as the key residue in determining the specificity of AspAT for dicarboxylic substrates (12) through direct salt bridge-hydrogen bond interactions with the β- or ω-carboxylate groups of the substrate. Indeed, replacement of Arg-292 with an aspartate residue has converted E. coli AspAT from an anionic to a very slow cationic amino acid transaminase (37). Similarly, substitution of Arg-292 with valine or leucine has been found to switch the specificity in favor of aromatic amino acids (38).

Recently, the importance of the guanidinium group of arginine has also been pointed out by the marked decrease in both the affinity and the catalytic activity toward dicarboxylic substrates upon replacement of Arg-283 with threonine (39). Here, in an attempt to change the substrate specificity of TPL from l-tyrosine to dicarboxylic substrates, we introduced an arginine residue in TPL in the same position that, as indicated by homology modeling, is occupied by Arg-292 in AspAT. Measurements of enzymic activities demonstrated indeed the conversion of TPL, a tyrosine β-lyase, to a dicarboxylic amino acid β-lyase. TPL R100T/V283R catalyzes the β-elimination reaction of l-lysine at a 2-fold higher rate than that of l-tyrosine, the rate of β-elimination of l-lysine being only 1 order of magnitude slower than that of β-elimination of l-tyrosine by the wild-type enzyme.

The dicarboxylic amino acids l-aspartate, l-glutamate, and l-2-aminoadipate are converted to pyruvate, ammonia, and the respective monocarboxylic acids, e.g. with l-aspartate as substrate, production of formate was observed. The pathway of formate production from l-aspartate by β-elimination corresponds to that followed by the wild-type enzyme with its natural substrate l-tyrosine (Scheme 1). Upon formation of the quinonoid intermediate 2, the coenzyme donates electrons to the substrate resulting in the cleavage of the bond between Cα and the distal carboxyl group thus producing the monocarboxylate coenzyme-substrate adduct 3. The cleavage of the aliphatic C=C bond very likely is facilitated by an active-site group which protonates the carbamic of the leaving carboxylic acid in a concerted fashion. Transamination of the monocarboxylate intermediate leads to the production of ammonium pyruvate 4, restoring the internal aldimine. The fact that no l-alanine was found in the reaction mixture together with the production of equimolar amounts of pyruvate and formate demonstrates that no β-decarboxylation occurred. Thus, β-elimination and slow transamination of l-lysine (Table III) are the only reactions taking place. Analogous results have been obtained with l-glutamate and l-2-aminoadipate as substrates. It has to be noted that the known PLP-dependent carbon-carbon lyases acting on dicarboxylic amino acids are decarboxylases, e.g. glutamate decarboxylase (40) and aspartate β-decarboxylase (41, 42), that produce CO₂ and the cognate monocarboxylic amino acid. The reaction observed here reflects a newly generated catalytic activity for PLP-dependent enzymes toward dicarboxylic amino acids. TPL R100T/V283R and TPL R100T are the first B₆ enzymes that catalyze the β-elimination reaction of these substrates.

The importance of the introduction of an arginine residue at position 283 for the recognition of dicarboxylic substrates is evident from a comparison of TPL R100T/V283R with TPL R100T. The single mutant TPL also catalyzes the β-elimination reaction of dicarboxylic substrates; however, the double mutant TPL R100T/V283R reacts faster with l-lysine, and its K₅₅ value for l-glutamate is lower (Table I). Moreover, the data agree with the hypothesis that Arg-100 is the positively charged group in the active site of TPL that interacts with the distal carboxylate group of l-lysine or l-glutamate making them potential inhibitors but not substrates of wild-type TPL. This mode of inhibition of TPL by dicarboxylic acids has been proposed previously by Faleev et al. (34). Replacement of Arg-100 with threonine renders dicarboxylic substrates more flexible in the active site. The additional introduction of Arg-283 might stabilize the side chain carboxylate group in a more favorable position for reaction due to salt bridge-hydrogen bond interactions similar to those in AspAT (Fig. 4).

Recent studies (43) have demonstrated that Arg-381 in TPL is required for the recognition of l-tyrosine as substrate. We found that not only the R381I enzyme but also the triple mutant TPL R100T/V283R/R381I has no measurable activity toward both l-tyrosine and dicarboxylic substrates (data not shown).

Apparently, dicarboxylic substrates adopt in the active site of TPL R100T/V283R a similar configuration as l-tyrosine in wild-type TPL and interact in a similar way with the critical residues of TPL that control reaction specificity. Even though the overall catalytic activity of TPL R100T/V283R toward l-tyrosine is decreased, its K₅₅ value for this substrate is almost unchanged (Table I) suggesting that the side chain of Arg-283 contributes to the binding of the aromatic ring of l-tyrosine. Such interactions have been reported to apply in E. coli aromatic amino acid aminotransferase (44). How does the side chain of Arg-283 contribute to the recognition of l-tyrosine?
The model of the "arginine switching" mechanism (45) assumes that the side chain of arginine moves out of the active site, when aromatic monocarboxylic substrates are bound. This model has been verified by x-ray crystallographic analysis of aspartate aminotransferase that has been engineered into a tyrosine aminotransferase (19, 46). Another model assumes that the guanidinium group of arginine directly links up with the aromatic ring of the bound substrates. This interaction may also cause the side chain of arginine to move out of the active site, when aromatic monocarboxylic substrates are bound. This model has been confirmed by the results of previous studies, which indicate that different active-site residues are important for the catalysis of β-elimination and transamination reactions.

In conclusion, the newly generated substrate specificity of TPL R100T/V283R agrees with previous studies in which the dicarboxylic amino acid β-lyase, i.e., enhancement of its substrate binding affinity and catalytic efficacy, would require substitutions of numerous amino acid residues that do not participate in the active site.

Acknowledgments—We are grateful to Prof. R. S. Phillips for useful discussions and for providing us with the plasmid pTZTPL, the expression system, and S-(o-nitrophenyl)-l-cysteine.

REFERENCES